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Abstract

Introduction

Systemic sclerosis (SSc) is a connective tissue disorder characterised by the development of skin fibrosis. Our current understanding of the disease pathogenesis is incomplete and the study of SSc is hindered, at least partially, by a lack of animal models that fully replicate the complex state of human disease. Murine model of bleomycin-induced dermal fibrosis encapsulates important events that take place early in the disease course.

Methods

To characterise the optimum *in vivo* parameters required for the successful induction of dermal fibrosis we subjected three commonly used mouse strains to repeated subcutaneous bleomycin injections. We aimed to identify the effects of genetic background and gender on the severity of skin fibrosis. We used male and female Balb/C, C57BL/6, and DBA/2 strains and assessed their susceptibility to bleomycin-induced fibrosis by measuring dermal thickness, hydroxyproline/collagen content and number of resident myofibroblasts, all of which are important indicators of the severity of skin fibrosis. All data are expressed as mean values ± SEM. The Mann–Whitney U test was used for statistical analysis with GraphPad Prism 6.04 software.

Results

Dermal fibrosis was most severe in Balb/C mice compared to C57BL/6 and DBA/2 suggesting that Balb/C mice are more susceptible to bleomycin-induced fibrosis. Analysis of the effect of gender on the severity of fibrosis showed that male Balb/C, C57BL/6, DBA/2 mice had a tendency to develop more pronounced fibrosis phenotype than female mice. Of potential importance, male Balb/C mice developed the most severe fibrosis phenotype compared to male C57BL/6 and male DBA/2 as indicated by significantly increased number of dermal myofibroblasts.

Conclusion

Our study highlights the importance of genetic background and gender in the induction of murine dermal fibrosis. Robust and reproducible animal models of fibrosis are important research tools used in pharmacological studies which may lead to better understanding of the pathogenesis of fibrotic diseases and assist in identification of new drugs.

Introduction

Systemic sclerosis (SSc) is an autoimmune disorder characterised by progressive connective tissue fibrosis and life-threatening complications with high mortality and morbidity [1]. It has long been known that the level of available transforming growth factor-β (TGF-β), connective tissue growth factor and other profibrotic molecules in the dermis are critical for the development and sustaining of fibrosis in SSc [2]. Furthermore, dermal fibrosis in SSc is thought to be the result of activation and differentiation of fibroblasts into apoptosis resistant and α-smooth muscle actin (α-SMA)-positive myofibroblasts. Increased expression of

myofibroblasts further stimulates the formation of extracellular matrix (ECM) leading to aberrant skin architecture and pathological tissue remodelling [3]. There are no mechanistic-based therapies, such as pharmaceutical drugs on the market that prevent and control the progression of excessive ECM formation in SSc. Thus, there is an urgent need to better understand fibrosis, devise processes for manipulating ECM formation and reduce excessive collagen deposition. The ability to develop novel anti-fibrotic therapies and analyse their efficacy in proof-of-concept (POC) studies is partly impeded by limitations in currently available animal models used to study this disorder *in vivo*. The pathophysiology of SSc is complex and believed to be caused by multiple factors including vasculopathy, inflammation, and autoimmunity [4,5]. Not surprisingly, there is currently no animal model that perfectly mimics all the steps associated with the development of dermal fibrosis. Although several inducible and genetic models exist [4,5], not a single one of these models recapitulates all of the clinical features consistent with human SSc [3]. Murine model of bleomycin-induced dermal fibrosis is widely used to study the changes that take place in the early phase of the disease [6]. Bleomycin is a glycopeptide-derived anti-tumor antibiotic, which was first isolated from a culture broth of *Streptomyces verticillus* [7]. Bleomycin induces the release of reactive oxygen species, recruitment of inflammatory cells, which activate resident fibroblasts and stimulate ECM formation. Due to its profibrotic properties, subcutaneous bleomycin is used to induce local skin fibrosis known to persist for up to six weeks [8]. Apart from its local effect, high dose subcutaneous bleomycin injections are thought to induce lung fibrosis [7] and systemic autoimmune responses characterised by the presence of antinuclear autoantibodies, such as anti–Scl-70, anti–U1 RNP [9].

The model of bleomycin-induced skin fibrosis, originally described by Yamamoto [10], has been used widely in preclinical [11] and pharmacological studies [12]. Several modifications of this protocol exist raising the issue of study-to-study variance of the resulting dermal fibrosis. Given that bleomycin-induced model of SSc is an important tool in understanding the pathogenesis of fibrotic skin changes, we investigated the susceptibility and intensity of dermal fibrosis observed in mouse skin of three widely utilised mouse strains of both genders. We therefore hypothesised that different strains of mice will have various degrees of sensitivity in response to bleomycin-induced dermal fibrosis. The aim of this study was to suggest an optimised protocol and describe the methods necessary for the induction of standardised model of fibrosis in adult mouse skin. The observations of this study are to be used as a guide to finding a suitable animal model, which is morphologically and histologically consistent with early stages of SSc-associated skin fibrosis.

Methods

Murine model of bleomycin-induced dermal fibrosis

Three mouse stains, namely Balb/C, C57BL/6, DBA/2 (Janvier, Genest-St-Isle, France), were used in the studies. Balb/C strain, originally created from a stock of outbred albino mice, was systematically inbred and has the following related genotype: Tyr^{c}/ Tyr^{c}, $Tyrp1^{b}$/$Tyrp1^{b}$, A /A - MHC: Haplotype $H2^{d}$. The inbred strain of C57BL/6 mice (related genotype: a (a/a) non agouti - MHC: Haplotype $H2^{b}$) is a widely used strain, which is frequently used as genetic background in transgenic models http://www.janvierlabs.com/tl_files/_media/images/FICHE_RESEARCH_MODEL_C57BL6 JRj.pdf. The DBA is the oldest of all inbred strains of mice, with the DBA/2 substrain having the following genotype*:* a/a, $Tyrp1^{b}$/$Tyrp1^{b}$, $Myo5a^{d}$/$Myo5a^{d}$ - MHC*:* Haplotype $H2^{d}$.

To determine whether gender and mouse strain have an influence on the severity of dermal fibrosis we subjected one group of male Balb/C (n=6), C57BL/6 (n=6), DBA/2 (n=6) mice of 6 weeks of age, weighing 20–25 g to bleomycin injections at a concentration of 0.5 mg/ml. The upper dorsa of mice were shaved and one square measuring 1 cm^2 was drawn on the midline using a marker. 100 µl of bleomycin (Laboratoire Roger Bellon, Neuilly-sur-Seine, France) at 0.5 mg/ml dissolved in PBS was administered every other day for 21 days. As part of the protocol, bleomycin injection sites were rotated. The first four injections were administered into four different corners of the square followed by the fifth injection given in the middle of the square. This protocol was continued until the day of post-mortem, at which point the marked square created on dorsal surface of mice was harvested, with one half of biopsy fixed in 4 % wt/vol. paraformaldehyde for histology and the other half snap frozen for molecular biology.

Another group of female Balb/C (n=6), C57BL/6 (n=6), DBA/2 (n=6) was subjected to bleomycin injections (100 µl of bleomycin at 0.5 mg/ml, every other day for 21 days). Age-matched control animals were treated with and equivalent dose of vehicle. Each experimental group consisted of 6 mice.

In a separated experiment, we aimed to assess whether frequency of bleomycin injections had the capacity to alter the severity of dermal fibrosis. We subjected one group of male C57BL/6 mice (n=6) of 6 weeks of age, weighing 20–25 g to bleomycin injections (0.5 mg/ml) given every day and another group (n=6) to bleomycin injections (0.5 mg/ml) given every other day for 21 days. In a different experiment, we endeavoured to determine the effect of bleomycin dosage (C57BL/6 mice, bleomycin at 0.5 mg/ml vs. 1 mg/ml administered every other day for 21 days) on the severity of dermal fibrosis. Mice were killed at 21 days after the first bleomycin experiment. 5 mm of lesional skin was harvested and fixed in 4 % wt/vol. buffered formalin and processed for histology. Four lesional skin biopsies (3 mm each) were snap frozen in liquid nitrogen and used for colorimetric assessment of collagen content (hydroxyproline assay). All animal experiments were approved by the local Animal Ethics Committee (Comité National de Réflexion Ethique sur l'Expérimentation Animale-34) and principles of laboratory animal care were followed.

Histology, immunohistochemistry and image analysis

Histological sections (4 µm) were cut from paraffin-embedded formalin fixed lesional skin tissue. Sections were stained with haematoxylin and eosin and images were captured at × 100 microscopic magnification. Histological dermal thickness was determined by manually drawing a straight line between the epidermis and adipose layer. Image analysis was performed using the Image J (freely available Java image processing program). Blinded measurements of histological slides by two independent assessors was performed.

4 µm paraffin-embedded formalin fixed samples of lesional skin were subjected to immunohistochemistry according to manufacturer's protocol (Dako, Carpinteria, California, USA). Skin sections were deparaffinised and followed by antigen retrieval blocked in Super Block IDetect Super Stain System HRP (ID Labs, London ON N4A 5 K2, Canada) for 10 minutes followed by incubation with 3 % H_2O_2 for 10 min to block endogenous peroxidase activity. Primary antibodies against α-SMA (1:500, Abcam), CD3 (1:50, Abcam), CD22 (1:100, Abcam) and CD68 (1:100, Abcam) was applied overnight. Species-specific, biotinylated secondary antibodies (1:200) were used and detection was by Dako Liquid DAB Chromagen System (Dako, Carpinteria, California, USA). Myofibroblasts were identified by

staining for α-SMA, as previously described [12]. The number of α-SMA, CD3, CD22 and CD68 positive cells was determined at high magnification in four different sections obtained from each mouse and determined by two blinded examiners. The number of α-SMA, CD3, CD22 and CD68 positive cells was counted and expressed as a number of total cells within each microscopic filed normalised against NaCl control as previously described [12]. Negative controls included replacing primary antibodies with normal species specific IgG.

Assessment of inflammatory infiltrate

Assessment of the number of infiltrating mononuclear/inflammatory cells in bleomycin and NaCl-treated mouse skin was determined using H&E stained sections as well as immunohistochemistry images of CD3, CD22 and CD68 stained skin as described previously [12]. Eight different high power fields from each mouse skin sections were evaluated for inflammatory infiltrate by two independent examiners blinded to the treatment.

Multiphoton microscopy

Multiphoton inverted stand Leica SP5 microscope (Leica Microsystems Gmbh, Wetzlar, Germany) was used for tissue imaging as previously described [13]. A Ti:Sapphire Chameleon Ultra (Coherent, Saclay, France) with a center wavelength at 810 nm was used as the laser source for generating second harmonic generation (SHG) and two-photon excited fluorescence signals (TPEF). The laser beam was circularly polarized and we used a Leica Microsystems HCX IRAPO 25x/0.95 W objective. SHG and TPEF signals were detected in epi-collection through a 405/15-nm and a 525/50 bandpass filters respectively, by NDD PMT detectors (Leica Microsystems) with a constant voltage supply, at constant laser excitation power, allowing direct comparison of SHG intensity values. LAS software (Leica, Germany) was used for laser scanning control and image acquisition.

Masson's trichrome staining

For direct visualisation of collagen fibres and histological assessment of collagen deposition, trichrome staining was performed using the Masson Trichrome Staining Kit (Sigma-Aldrich, St Louis, MO, USA). Skin sections stained with Masson Trichrome were visualised at × 200 microscopic magnification. All images were captured with Olympus BX63F microscope (Olympus, Tokyo, Japan) equipped with a digital signal processor CoolSNAP scientific CCD camera (Photometrics, Tucson, AZ, USA).

Collagen measurements

The collagen content in lesional skin samples was explored by hydroxyproline assay. After digestion of punch biopsies (3 mm) in 6 M HCl for three hours at 120 °C, the pH of the samples was adjusted to 7 with 6 M NaOH. Samples were then mixed with 0.06 M chloramine T and incubated for 20 min at room temperature. Next, 3.15 M perchloric acid and 20 % p-dimethylaminobenzaldehyde were added and samples were incubated for additional 20 min at 60 °C. The absorbance was determined at 557 nm with a Spectra MAX 190 micro plate spectrophotometer (Molecular Devices, Sunnyvale, California, USA).

Statistics

All data are expressed as mean values ± SEM. The Mann–Whitney U test for non-related samples was used for statistical analysis with GraphPad Prism 6.04 software (San Diego, CA). A p value of less than 0.05 was considered statistically significant.

Results

Pro-fibrotic effects of subcutaneous bleomycin injections in the dermis of male Balb/C, C57BL/6 and DBA/2 mice

To determine whether bleomycin had the same capacity to induce dermal fibrosis in three (Balb/C, C57BL/6 and DBA/2) inbred mouse strains that are frequently used for studies in the field of dermal fibrosis research, we administered bleomycin at a concentration of 0.5 mg/ml, which was injected every other day for the period of 3 weeks. Assessment of dermal thickness, hydroxyproline content and myofibroblast count showed that bleomycin successfully induced dermal fibrosis in all of the three strains assessed (Fig. 1a-f). Bleomycin treatment in Balb/C, C57BL/6 and DBA/2 mice was associated with an increase in dermal thickness by 19.89 %, 21.31 % and 18.7 % respectively when compared to NaCl-treated counterparts (Fig. 1b). No significant difference in dermal thickness was found between bleomycin treated male Balb/C, C57BL/6 and male DBA/2 mice (Fig. 1b). To confirm that the observed increase in dermal thickness in bleomycin-treated skin was due to increased collagen content, type I fibrillary collagen has been imaged by SHG (Fig. 1c) and hydroxyproline assay (Fig. 1d) were performed. Note similar baseline distribution of collagen fiber alignment in NaCl-treated Balb/C, C57BL/6 and DBA/2 mice (Fig. 1c, d). Consistent with increased dermal thickness in bleomycin treated groups, increased accumulation of collagen was observed in Balb/C, C57BL/6 and DBA/2 skin when compared to NaCl-treated controls (Fig. 1c; $p = 0.0290$ in Balb/C; $p = 0.0001$ in C57BL/6; $p = 0.0103$ in DBA/2).

Fig. 1 Histological and histochemical analysis of dermal fibrosis in male bleomycin-treated Balb/C, C57BL/6 and DBA/2 mice. **a** Representative images of H&E-stained sections of male mouse skin treated with subcutaneous NaCl or bleomycin given every other day over a period of 3 weeks. Original magnification ×100. **b** Graphical representation of dermal thickness of skin harvested from mice treated with NaCl or bleomycin after 3 weeks. Four high power field images were taken (2 measurements per image). Results represent the relative fold change compared to NaCl-treated control mice. All values represent means ±SEM; n=6 each group. **c** Representative images of SHG imaging of type I fibrillary collagen in mouse skin harvested after 3 weeks of treatment with NaCl or bleomycin injected every other day. Note different distribution of fiber alignment and intensity in NaCl and bleomycin treated samples. We note that all images were acquired with the same excitation power. Original magnification ×250. **d** Graphical representation of hydroxyproline assay. Results are represented as means ±SEM of triplicate measurements obtained from 6 mice (2 biopsies per mouse) and shown as relative fold change compared to NaCl-treated control samples. **e** Representative images of α-SMA immunohistochemistry. Original magnification ×200, inset ×630. **f** Graphical representation of relative number of α-SMA-positive cells in dermis of NaCl or bleomycin-treated mice. Results represent the relative fold change compared to NaCl-treated control mice. All values represent means ±SEM; n=6 each group

Increased levels of collagen content in bleomycin treated skin may result from increased recruitment of myofibroblasts. In order to determine whether myofibroblasts contributed to increased levels of collagen in the dermis of male Balb/C, C57BL/6 and DBA/2 mouse skin, we analysed the number of myofibrolbasts (Fig 1e-f). Under fibrotic conditions myofibroblast count increased by 5-fold in male Balb/C compared to NaCl control, by 2-fold in male C57BL/6 and 3-fold in male DBA/2 mice. Male Balb/C mice treated with bleomycin showed significantly increased count of dermal myofibroblasts compared to bleomycin-treated male C57BL/6 mice ($p = 0.0041$) and DBA/2 mice ($p = 0.05$) (Fig 1f).

Female Balb/C mice are more 'susceptible' to bleomycin-induced dermal fibrosis than female C57BL/6 and DBA/2 counterparts

Dermal fibrosis was induced in female Balb/C, C57BL/6 and DBA/2 via subcutaneous bleomycin injections given for 3 weeks at a final concentration of 0.5 mg/kg and given every other day. Histological changes were determined by measuring the length of dermal thickness in bleomycin-treated and NaCl control female Balb/C, C57BL/6 and DBA/2 mice. Dermal thickness was determined by measuring the average thickness between the epidermal-dermal and dermal–subcutaneous fat junctions.

Bleomycin treatment resulted in an increase of dermal thickness in female Balb/C mice compared to NaCl-treated female Balb/C mice (Fig. 2b; $p = 0.0001$). No significant difference was observed in dermal thickness of bleomycin-treated female C57BL/6 mice compared to NaCl-treated female controls (Fig. 2b), however there was a 43.7 % ($p=0.0036$) increase in collagen content and 33.8 % increase ($p=0.0136$) in myofibroblast count in female C57BL/6 mice compared to NaCl-treated female controls (Fig. 2c-d). There was no significant change in dermal thickness, hydroxyproline content and myofibroblast count in female DBA/2 mice treated with bleomycin compared to female DBA/2 mice treated with NaCl (Fig. 2a-f).

Fig. 2 Female Balb/C mice are more susceptible to bleomycin-induced dermal fibrosis than female C57BL/6 and DBA/2 mice. **a** Representative images of H&E-stained sections of female mouse skin treated with subcutaneous NaCl or bleomycin injections given every other day over a period of 3 weeks. Original magnification ×100. **b** Graphical representation of dermal thickness of female mouse skin harvested from mice treated with NaCl or bleomycin after 3 weeks. Four high power field images were taken (2 measurements per image). Results represent the relative fold change compared to NaCl-treated control mice. **c** Representative images of SHG imaging of type I fibrillary collagen in mouse skin harvested after 3 weeks of treatment with NaCl or bleomycin injected every other day. Original magnification ×250. **d** Graphical representation of hydroxyproline assay. Results are represented as means ±SEM of triplicate measurements obtained from 6 mice (2 biopsies per mouse) and shown as relative fold change compared to NaCl-treated control samples. **e** Representative images of α-SMA immunohistochemistry. Original magnification ×200, inset ×630. **f** Graphical representation of relative number of α-SMA-positive cells in dermis of NaCl or bleomycin-treated mice. Results represent the relative fold change compared to NaCl-treated control mice. All values represent means ±SEM; n=6 each group

Effect of gender on the induction of experimental dermal fibrosis

To study the effect of gender on the induction of dermal fibrosis we compared fibrotic phenotype as determined by three parameters: dermal thickness, hydroxyproline content and myofibroblast count. Although male Balb/C and male C57BL/6 mice have scored higher in histological and immunohistochemical analysis of bleomycin treated skin, there was no significant difference in dermal thickness, hydroxyproline and myofibroblast count between male and female Balb/C and C57BL/6 mice (Fig. 3a-c). The number of myofibroblasts in male DBA/2 mice was significantly higher than in female DBA/2 mice (Fig. 3c; p=0.0355).

Fig. 3 Gender effects the severity of dermal fibrosis and inflammatory response. **a** Bleomycin-treated male Balb/C and male DBA/2 mice have slightly higher dermal thickness and **b** hydroxyproline content compared to their female counterparts, however this number doesn't reach significance. **c** Bleomycin-treated male DBA/2 mice have significantly higher myofibroblasts than their female counterparts. **d** Representative images of H&E-stained sections of male Balb/C, C57BL/6 and DBA/2 skin subjected to bleomycin (0.5 mg/ml) injections given every other day. **e** Graphical representation of relative number of inflammatory cells in dermis of male Balb/C, C57BL/6 and DBA/2 in response to bleomycin injections**. f** Representative images of H&E-stained sections of female Balb/C, C57BL/6 and DBA/2 skin subjected to bleomycin (0.5 mg/ml) injections given every other day. **g** Graphical representation of relative number of inflammatory cells in dermis of female Balb/C, C57BL/6 and DBA/2 in response to bleomycin injections**.** Original magnification ×630. All values represent means ±SEM; n=6 each group

Male DBA/2 mice have the lowest number of infiltrating leukocytes

In addition to fibrotic skin changes, repetitive bleomycin injections are known to cause skin inflammation as defined by increased number of leukocytes recruited to the lesional area [7]. To determine whether the type of mouse strain had an effect on the number of inflammatory cells and the rate of inflammation in bleomycin-treated skin (0.5 mg/ml, alternate days, 3 weeks), male and female Balb/C, C57BL/6 and DBA/2 skin was stained with H&E and the number of leukocytes was counted (Fig. 3d and e). The number of leukocytes in bleomycin-treated male Balb/C and C57BL/6 skin was 2.1 and 2.5-fold higher respectively than in bleomycin-treated male DBA/2 mice (Fig. 3d and e; p=0.0142 and p=0.0018 respectively). The type of mouse strain did not have an effect on the rate of inflammation in females since there was no significant difference in the number of leukocytes between female Balb/C, female C57BL/6 and female DBA/2 mice (Fig. 3e and g).

To identify the nature of infiltrating leucocytes and determine the influence of mouse strain on leucocyte infiltration, we next quantified the number of CD3, CD22 and CD68 positive cells in male and female Balb/C, C57BL/6 and DBA/2 skin. The number of CD3, CD22 and CD68 positive cells were significantly reduced in male DBA/2 bleomycin-treated skin compared to bleomycin treated male Balb/C and C57BL/6 skin (Fig. 4a-f). There appeared to be no difference between the number of CD3, CD22 and CD68 positive cells in bleomycin challenged female Balb/C, C57BL/6 and DBA/2 skin. (Fig. 4g-l).

Fig. 4 Male DBA-2 mice showed reduced number of CD3, CD22 and CD68 cell infiltration into bleomycin challenged mouse skin. Representative images of (**a**) CD3, (**b**) CD22 and (**c**) CD68 immunohistochemistry sections of male Balb/C, C57BL/6 and DBA/2 skin subjected to bleomycin (0.5 mg/ml) injections given every other day. **d-f** Graphical representation of relative number of CD3, CD22 and CD68 positive cells in dermis of male Balb/C, C57BL/6 and DBA/2 in response to bleomycin injections**.** Original magnification ×400. All values represent means ±SEM; n=6 each group. Representative images of (**g**) CD3, (**h**) CD22 and (**i**) CD68 immunohistochemistry sections of female Balb/C, C57BL/6 and DBA/2 skin subjected to bleomycin (0.5 mg/ml) injections given every other day. (**j-l**) Graphical representation of relative number of CD3, CD22 and CD68 positive cells in dermis of female Balb/C, C57BL/6 and DBA/2 in response to bleomycin injections. Original magnification ×400. All values represent means ±SEM; n=6 each group

Effect of frequency of bleomycin administration on the severity of mouse skin fibrosis

To establish whether the frequency of bleomycin injections had an effect on the severity of skin fibrosis, male C57BL/6 mice were given daily subcutaneous injections of bleomycin (at 0.5 mg/ml, 3 weeks) and compared to male C57BL/6 mice which were given bleomycin injections on alternate days (at 0.5 mg/ml, 3 weeks). In response to bleomycin, which was given either daily or on alternate days, dermal thickness increased in bleomycin-treated mice compared to NaCl-treated controls (Fig. 5a and b). Although there was no difference in dermal thickness between mice treated with bleomycin daily or on alternate day (Fig. 5b), collagen accumulation as determined by hydroxyproline content (Fig. 5d, $p=0.0173$) was significantly elevated in mice treated with bleomycin on alternate days compared to those treated every day. While myofibroblast count was slightly higher in mice treated with bleomycin on alternate days compared to those treated every day, this number did not reach significance (Fig. 5f).

Fig. 5 Male C57BL/6 mice subjected to bleomycin injections on alternate days show higher dermal collagen content than male C57BL/6 mice given daily bleomycin injections. **a** Representative images of H&E-stained sections of male C57BL/6 mouse skin treated with subcutaneous NaCl or bleomycin injections given either daily or every other day over a period of 3 weeks. Original magnification ×100. **b** Graphical representation of dermal thickness of male C57BL/6 mouse skin treated with subcutaneous NaCl or bleomycin injections given either daily or every other day over a period of 3 weeks. Four high power field images were taken (2 measurements per image). Results represent the relative fold change compared to NaCl-treated control mice. **c** Representative images of Masson's Trichrome-stained sections of mouse skin harvested after 3 weeks of treatment with NaCl or bleomycin injected either every day or every other day. Original magnification ×100. **d** Graphical representation of hydroxyproline assay which was used as an indicator of collagen content in mouse skin treated with bleomycin every day vs. alternate days. Results are represented as means ±SEM of triplicate measurements obtained from 6 mice (2 biopsies per mouse) and shown as relative fold change compared to NaCl-treated control samples. **e** Representative images of α-SMA immunohistochemistry. Original magnification ×200, inset ×630. **f** Graphical representation of relative number of α-SMA-positive cells in dermis of mice treated with NaCl or bleomycin wither every day or alternate days. Results represent the relative fold change compared to NaCl-treated control mice. All values represent means ±SEM; n=6 each group

Increasing bleomycin concentration from 0.5 mg/ml to 1 mg/ml does not increase collagen deposition and myofibroblast accumulation in dermal fibrosis

Male C57BL/6 mice were given subcutaneous injections of bleomycin at a final concentration of 0.5 mg/ml or 1 mg/ml (alternate days, 3 weeks). Increasing bleomycin concentration from 0.5 mg/ml or 1 mg/ml did not have an effect on the severity of dermal fibrosis. No difference in dermal thickness (Fig. 6a, b), hydroxyproline content (Fig. 6c, d) and myofibroblast count (Fig. 6e, f) was found between mouse skin injected with bleomycin at 0.5 mg/ml vs. 1 mg/ml.

Fig. 6 The effect of low (0.5 mg/ml) and high (1 mg/ml) dosage of subcutaneous bleomycin injections on dermal thickness, collagen content and number of myofibroblasts in the skin of male C57BL/6 mice subjected to bleomycin on alternate days for 3 weeks. **a** Representative images of H&E-stained sections of male C57BL/6 mouse skin treated with subcutaneous NaCl or bleomycin injections (0.5 mg/ml or 1 mg/ml) given every other day for 3 weeks. Original magnification ×100. **b** Graphical representation of dermal thickness of male C57BL/6 mouse skin treated with subcutaneous NaCl or bleomycin injections (0.5 mg/ml or 1 mg/ml) given every other day for 3 weeks. Four high power field images were taken (2 measurements per image). Results represent the relative fold change compared to NaCl-treated control mice. **c** Representative images of Masson's Trichrome-stained sections of mouse skin harvested after 3 weeks of treatment with NaCl or bleomycin injections (0.5 mg/ml or 1 mg/ml) given every other day for 3 weeks. Original magnification ×100. **d** Graphical representation of hydroxyproline content in skin treated with low and high (0.5 mg/ml or 1 mg/ml) dosages of bleomycin. Results are represented as means ±SEM of triplicate measurements obtained from 6 mice (2 biopsies per mouse) and shown as relative fold change compared to NaCl-treated control samples. **e** Representative images of α-SMA immunohistochemistry in mouse skin treated with subcutaneous NaCl or bleomycin injections (0.5 mg/ml or 1 mg/ml) given every other day for 3 weeks. Original magnification ×200, inset ×630. **f** Graphical representation of relative number of α-SMA-positive cells in dermis of mice treated with NaCl or bleomycin (0.5 mg/ml vs. 1 mg/ml) on alternate days. Results represent the relative fold change compared to NaCl-treated control mice. All values represent means ±SEM; n=6 each group

Discussion

Bleomycin-treated Balb/C mice have the most severe fibrosis phenotype compared to C57BL/6 and DBA/2 suggesting that the severity of skin fibrosis, apart from other factors such as the route of administration and dose of bleomycin [14], also depends on genetic background of mice. Male Balb/C mice showed a greater number of myofibroblasts than C57BL/6 and DBA/2 of the same gender. Female Balb/C mice were previously shown to have higher susceptibility to bleomycin with greater dermal thickness measurements than their female C57BL/6 and DBA/2 counterparts [14]. Similarly, in this study female Balb/C mice showed greater levels of skin fibrosis characterised by increased dermal thickness, greater deposition of collagen and elevated number of dermal myofibroblasts, suggesting that, at least in the females, Balb/C mice may be more susceptible to bleomycin-induced skin fibrosis than their C57BL/6 and DBA/2 counterparts.

To determine the effect of gender on the severity of skin fibrosis, Balb/C, C57BL/6 and DBA/2 mice of both genders were used to induce and assess the extent of fibrosis. Male mice tended to have a more pronounced fibrosis phenotype than females and, as observed in DBA/2 mice, had significantly elevated number of myofibroblasts than female C57BL/6 and DBA/2 mice. The fact that male mice are more susceptible to the development of SSc-associated skin fibrosis is in agreement with the epidemiological observations which suggest that although women have increased susceptibility to SSc than men, males are known to have a more severe skin fibrosis phenotype [3,15]. Age is another factor which may have impacted our study. Human studies indicate that most women are diagnosed with SSc later in life and generally after the onset of menopause [16], whereas animals used in the current study were young and sexually active mice of both genders. Therefore, differences in the severity of fibrosis observed between the genders were possibly due to age, which in turn is characterised by differential expression of sex hormones. For instance, oestrogen was previously shown to have an influence on the development of SSc-associated fibrosis [17] with low levels of oestrogen being associated with severe fibrosis and exacerbated pulmonary hypertension [17]. In women oestrogens are at their lowest after the menopause and highest during pregnancy. Given that most women are diagnosed with SSc at the time when their oestrogen levels are at their lowest [16] and some autoimmune diseases, including rheumatoid arthritis [18] go into remission when oestrogens are at their peak, suggests that oestrogen may play an important protective role in autoimmune diseases such as SSc. Consistent with the observations in human SSc, increased circulating levels of oestrogen in young female compared to young male mice may have had a protective effect against the development of fibrosis offering a plausible explanation as to why male mice developed a more severe fibrosis phenotype than female mice.

Studying the characteristics of skin changes that develop as a result of repetitive bleomycin injections [10] can help to better understand inflammation and fibrosis, both of which are key elements that recapitulate the pathological events of SSc. Increased inflammatory infiltrate in lesional SSc skin, predominantly CD4+ T cells [19], suggests a distinct role of CD4 lymphocytes in the development of skin fibrosis. In animal experiments, bleomycin evokes pro-inflammatory response and increased prevalence of leukocytes. Due to its complexity and the involvement of multiple organs, SSc is difficult to replicate *in vivo*. While many animal models of SSc exist [4] there is currently no animal model that can capture this complexity. Along with its disadvantages, namely the absence of vascular complications [5], bleomycin-induced model of skin fibrosis provides us with an opportunity to study inflammation, elucidate the pathophysiology of SSc and explore potential treatment interventions. Inflammatory cells are thought to contribute to the initial activation of resident fibroblasts by the release of profibrotic mediators. To assess the contribution of genetic background on the development of inflammation associated with dermal fibrosis, Balb/C, C57BL/6 and DBA/2 of both genders were treated for 3 weeks with subcutaneous injection of bleomycin. Given that male DBA/2 mice showed the least number of CD3, CD22 and CD68 positive inflammatory cells, we suggest that in studies that focus on investigating the effect of inflammation on the pathogenesis of dermal fibrosis, strains other than DBA/2 are used. The number of inflammatory cells in bleomycin-treated male C57BL/6 mice was three times as much as the number of leucocytes found in DBA/2 mice, suggesting that C57BL/6 could be a strain of choice in studies concerning inflammation.

Having established the fact that male mice are more susceptible to dermal fibrosis than females we used male C57BL/6 mice to investigate if varying the frequency of bleomycin injections (every day versus alternate days) will have an effect on the severity of dermal

fibrosis. Bleomycin stimulates extracellular matrix formation by virtue of its profibrotic effects, and of importance, our studies indicated that daily exposure to bleomycin blunted the pro-fibrotic response of bleomycin in the skin, whereas alternate day administration enhanced this effect. Bleomycin injections administered on alternate days resulted in increased basal hydroxyproline, a biochemical marker of collagen, compared to daily injections. Local bleomycin injections are associated with active stimulation of leukocyte infiltration [20] driving the release of a plethora of proinflammatory cytokines including TGF-β1, which in turn promotes the synthesis and secretion of collagen and other matrix molecules [21]. Enhanced immune response and consequent TGF-β activation aggravate fibrosis, as it sets up a positive-feedback which could partially explain the reason why bleomycin injections given on alternate days are more efficient in stimulating the extracellular matrix. Alternate day injections may cause peaks of cytokine release in the skin and stimulate a more sustained inflammatory response than bleomycin injections given daily. The frequent peaks of proinflammatory cytokine release induced by bleomycin injections administered on alternated days versus daily injections might be important in mediating an efficient profibrotic effect of bleomycin. Bleomycin increased skin collagen levels in a dose-dependent manner and, although doses ≤ 0.5 mg/ml were reported to induce histological changes [10], our studies suggested that fibrotic responses were similar in mice treated with low (0.5 mg/ml) and high (1 mg/ml) doses of bleomycin.

Conclusions

In this study we evaluated the potential for mouse genetic background and gender to effect the induction of experimental mouse dermal fibrosis. With the information present herein, we suggest that dermal fibrosis studies are best done in male rather than female mice due to higher responsiveness to bleomycin injections. In C57BL/6 strain optimum results are obtained when treated with subcutaneous bleomycin at a final concentration of 0.5 mg/ml and administered on alternate days. These observations are of considerable importance with regard to the selection of an appropriate protocol necessary for the induction of dermal fibrosis, which may be used in pharmacological testing and therapeutical interventions.

Abbreviations

ECM, extracellular matrix; H&E, haematoxylin and eosin; HRP, horseradish peroxidase; SHG, second harmonic generation; SSc, systemic sclerosis; TGF-β, transforming growth factor beta; α-SMA, alpha smooth muscle actin.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JA, ME, MF and BR carried out the animal studies, histological assessment, helped to perform statistical analysis and revised the manuscript. NR and CF carried out histological and immunohistochemical analysis, performed statistical analysis and revised the manuscript. NR, JHD and YA conceived of the study, participated in its design and coordination and

revised the manuscript. NR drafted the manuscript. All authors read and approved the final manuscript.

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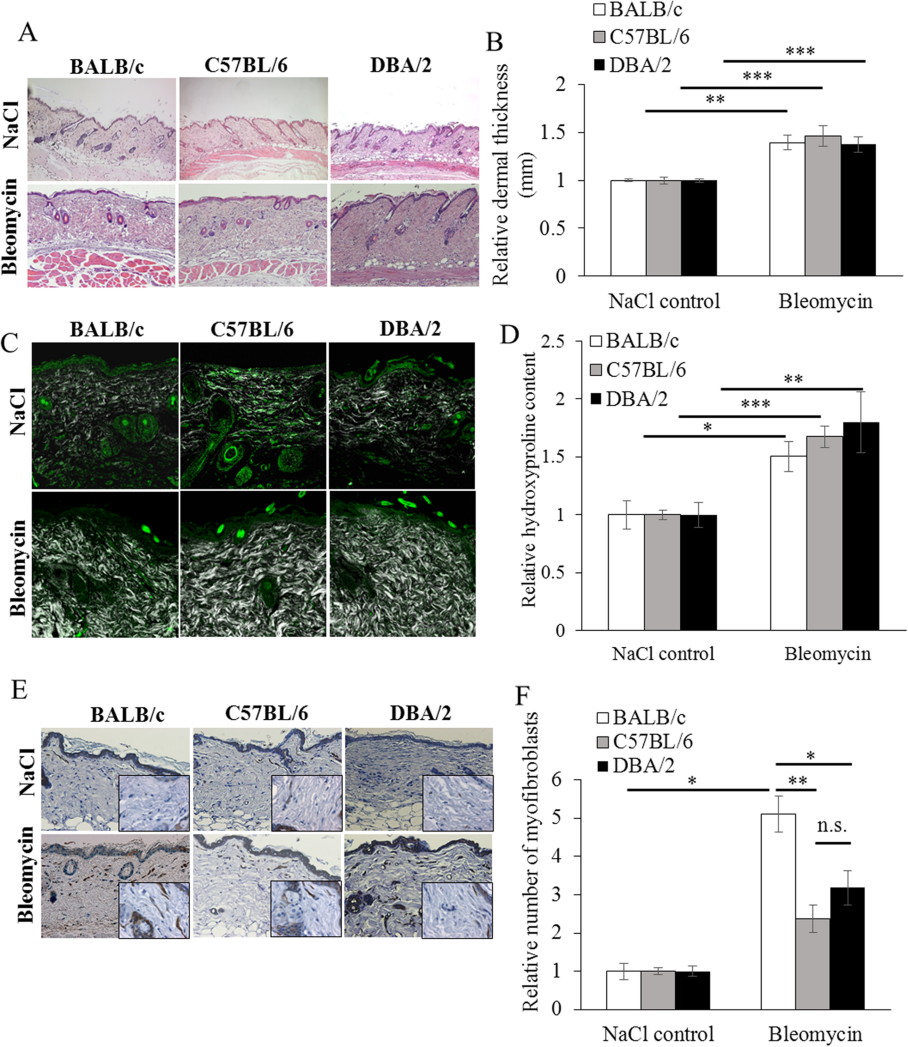
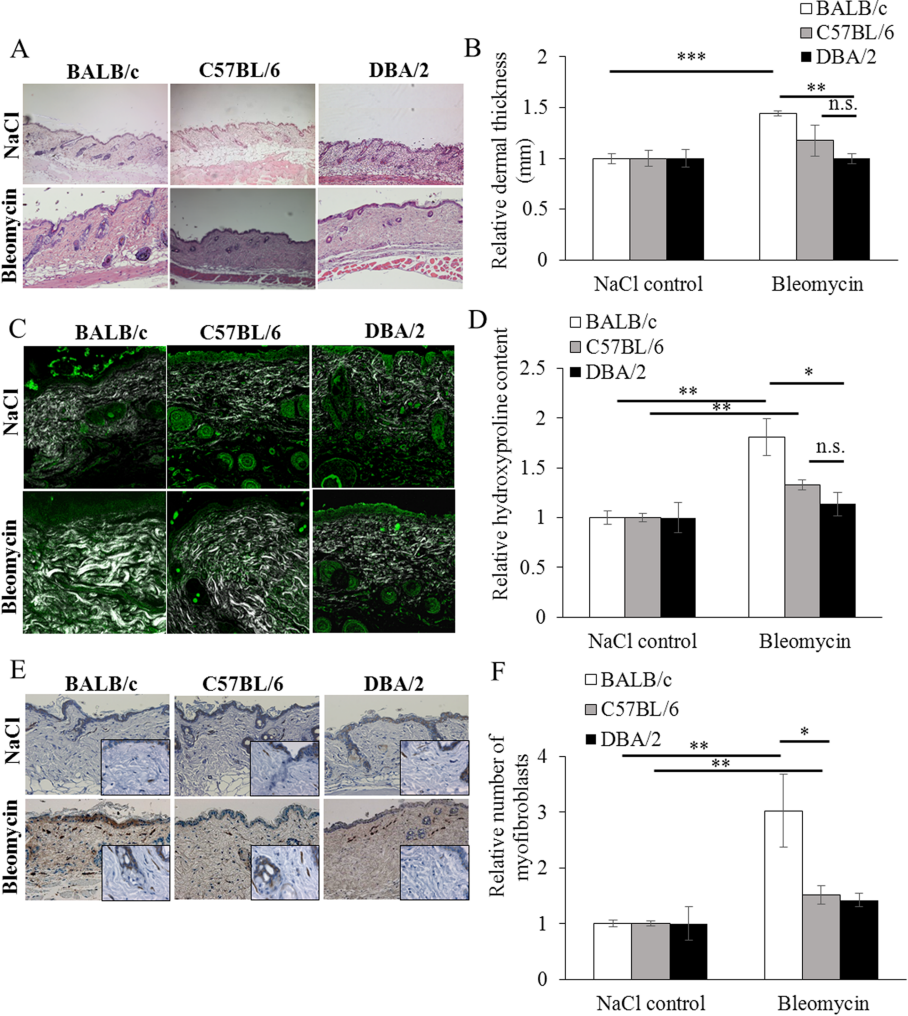
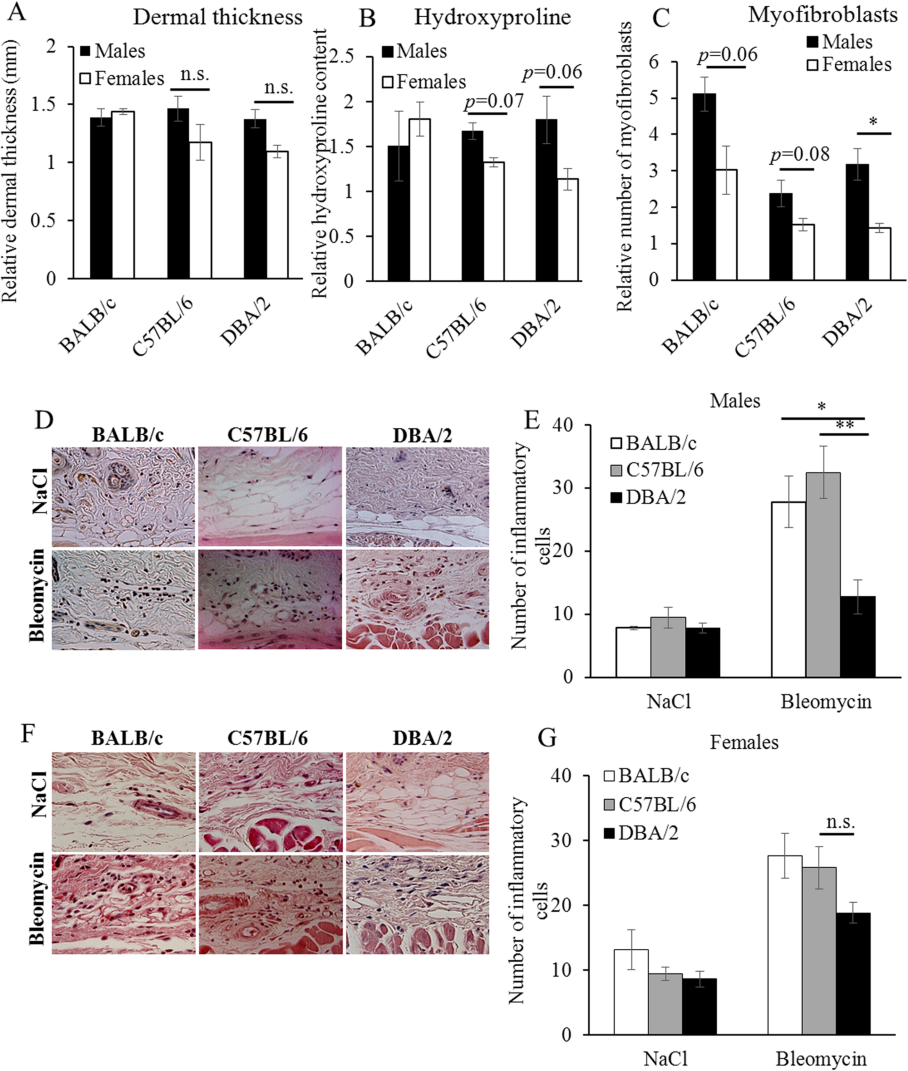
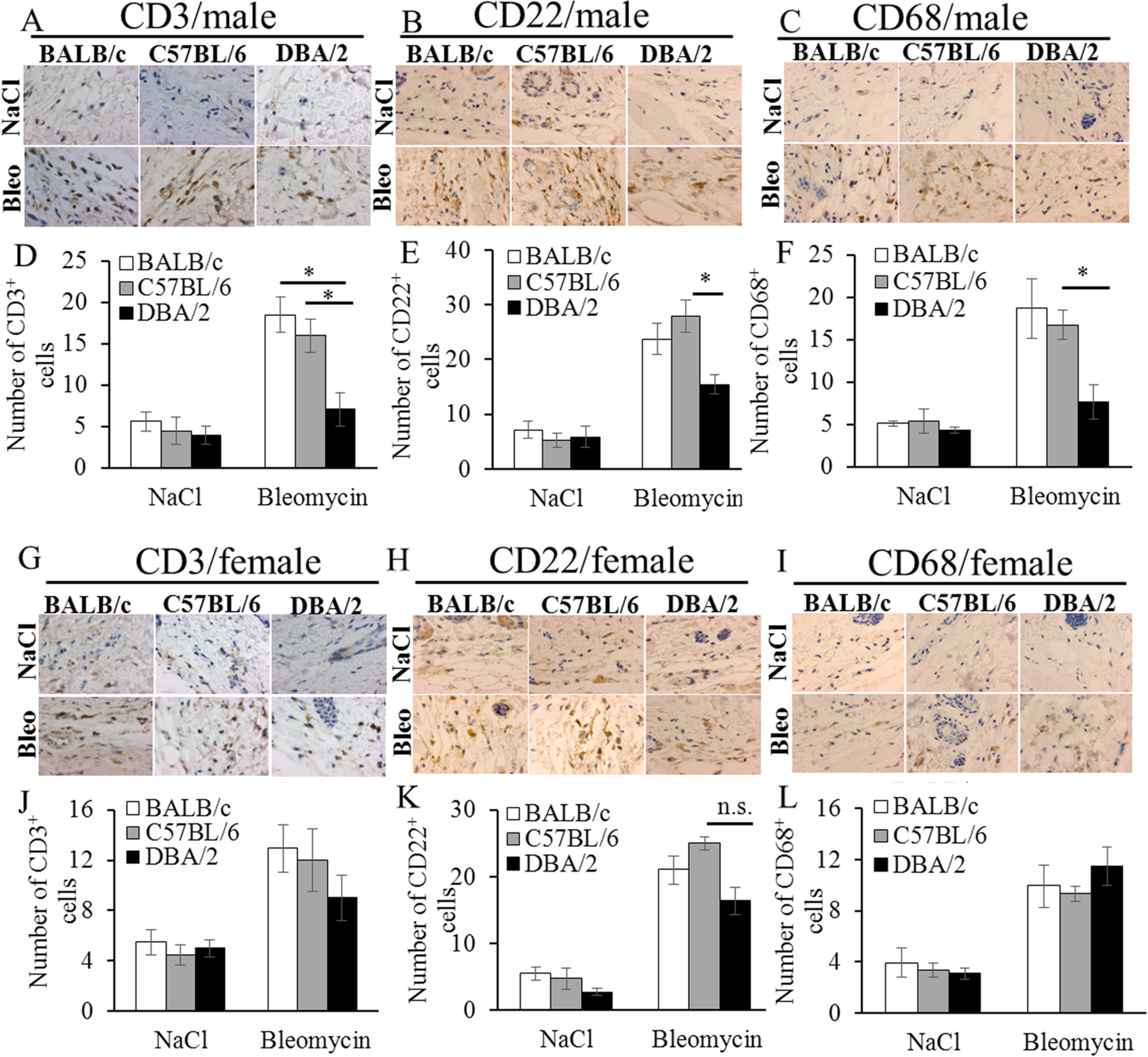
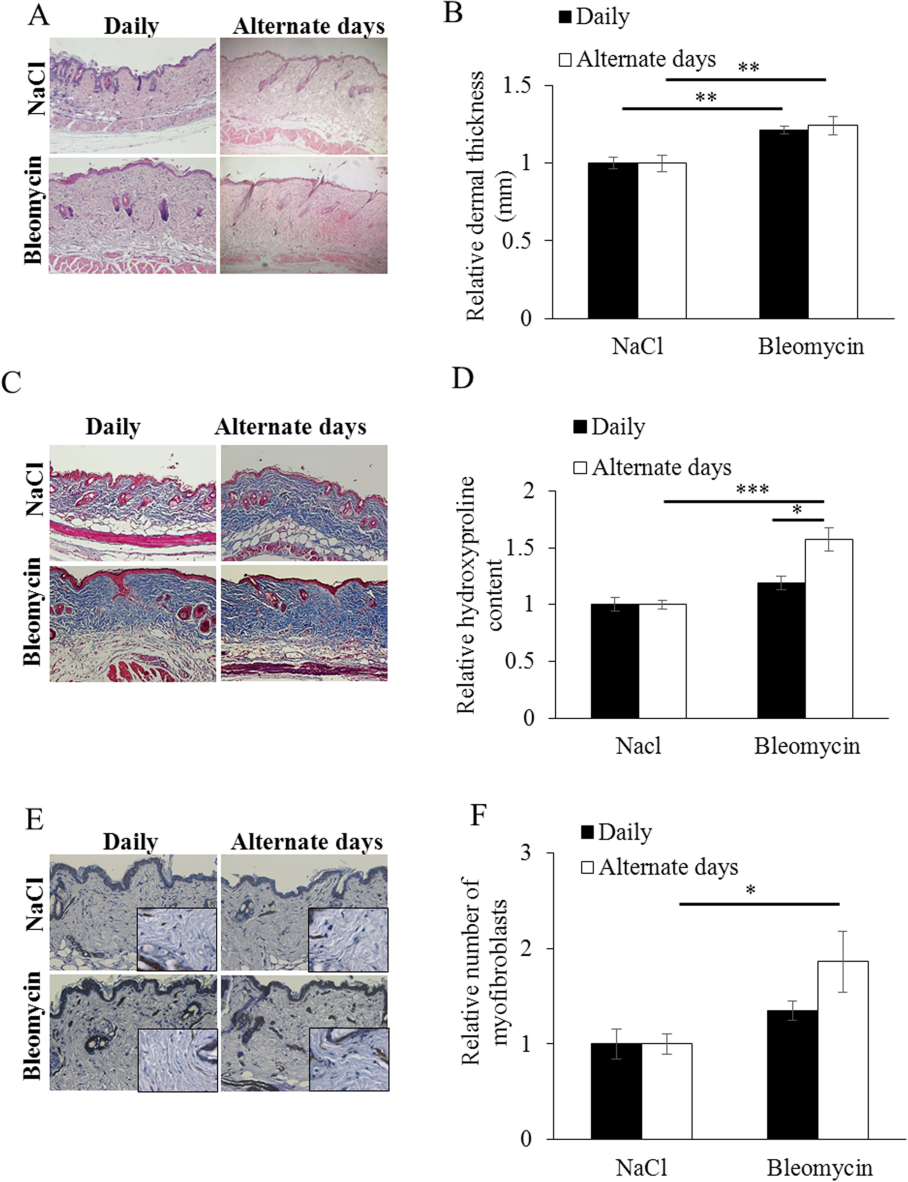
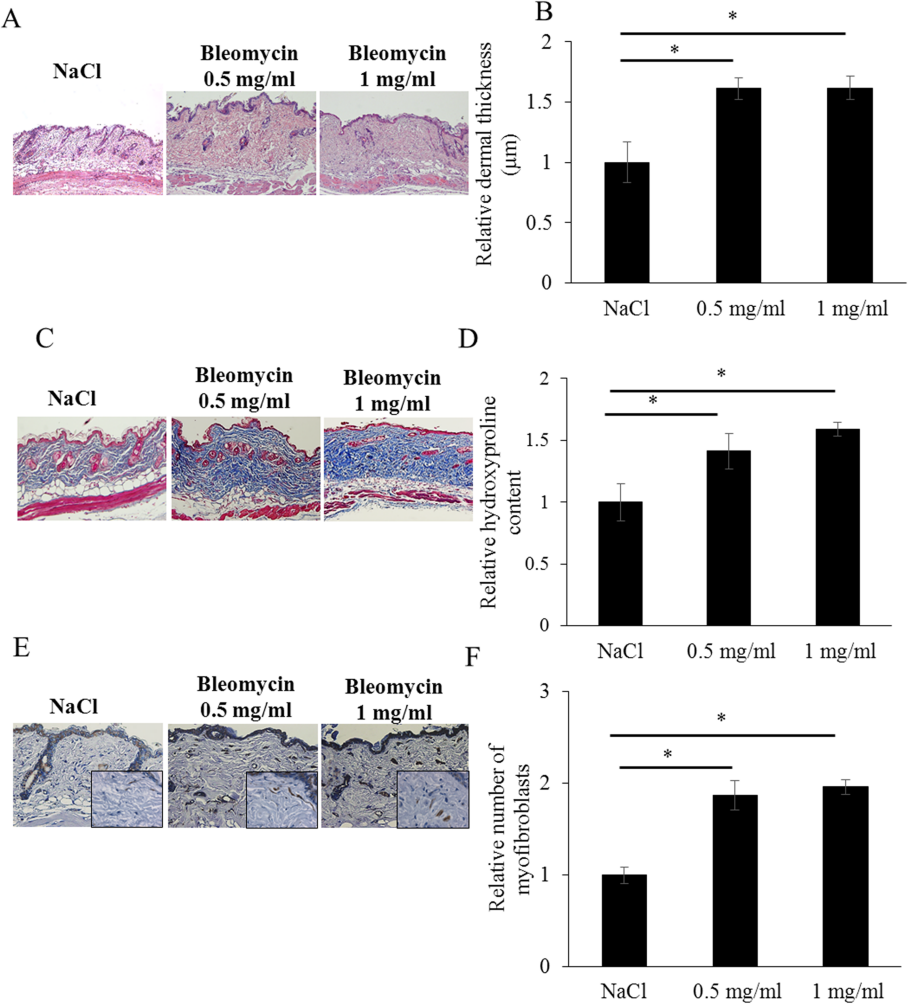

A
BALB/c
C57BL/6
DBA/2
NaCl
Bleomycin
B
BALB/c
C57BL/6
DBA/2
Relative dermal thickness (mm)
***
***
**
NaCl control
Bleomycin
C
BALB/c
C57BL/6
DBA/2
NaCl
Bleomycin
D
BALB/c
C57BL/6
DBA/2
Relative hydroxyproline content
**
***
*
NaCl control
Bleomycin
E
BALB/c
C57BL/6
DBA/2
NaCl
Bleomycin
F
BALB/c
C57BL/6
DBA/2
Relative number of myofibroblasts
*
*
**
n.s.
NaCl control
Bleomycin


A
BALB/c
C57BL/6
DBA/2
NaCl
Bleomycin
B
BALB/c
C57BL/6
DBA/2
Relative dermal thickness (mm)
***
**
n.s.
NaCl control
Bleomycin
C
BALB/c
C57BL/6
DBA/2
NaCl
Bleomycin
D
BALB/c
C57BL/6
DBA/2
Relative hydroxyproline content
**
**
*
n.s.
NaCl control
Bleomycin
E
BALB/c
C57BL/6
DBA/2
NaCl
Bleomycin
F
BALB/c
C57BL/6
DBA/2
Relative number of myofibroblasts
**
**
*
NaCl control
Bleomycin


A
Dermal thickness
Males
Females
n.s.
n.s.
Relative dermal thickness (mm)
BALB/c
C57BL/6
DBA/2
B
Hydroxyproline
Males
Females
p=0.07
p=0.06
Relative hydroxyproline content
C
Myofibroblasts
Males
Females
p=0.06
p=0.08
*
Relative number of myofibroblasts
D
BALB/c
C57BL/6
DBA/2
NaCl
Bleomycin
E
Males
*
**
BALB/c
C57BL/6
DBA/2
Number of inflammatory cells
NaCl
Bleomycin
F
BALB/c
C57BL/6
DBA/2
NaCl
Bleomycin
G
Females
BALB/c
C57BL/6
DBA/2
n.s.
Number of inflammatory cells
NaCl
Bleomycin


A CD3/male
B CD22/male
C CD68/male
BALB/c
C57BL/6
DBA/2
NaCl
Bleo
D
E
F
Number of CD3+ cells
Number of CD22+ cells
Number of CD68+ cells
BALB/c
C57BL/6
DBA/2
*
*
*
*
NaCl
Bleomycin
G CD3/female
H CD22/female
I CD68/female
J
K
L
n.s.


A
Daily
Alternate days
NaCl
Bleomycin
B
Daily
Alternate days
**
**
Relative dermal thickness (mm)
1.5
1
0.5
0
NaCl
Bleomycin
C
Daily
Alternate days
NaCl
Bleomycin
D
Daily
Alternate days
***
*
Relative hydroxyproline content
2
1.5
1
0.5
0
Nacl
Bleomycin
E
Daily
Alternate days
NaCl
Bleomycin
F
Daily
Alternate days
*
Relative number of myofibroblasts
3
2
1
0
NaCl
Bleomycin


A
NaCl
Bleomycin 0.5 mg/ml
Bleomycin 1 mg/ml
B
Relative dermal thickness (μm)
2
1.5
1
0.5
0
*
*
NaCl
0.5 mg/ml
1 mg/ml
C
NaCl
Bleomycin 0.5 mg/ml
Bleomycin 1 mg/ml
D
Relative hydroxyproline content
2
1.5
1
0.5
0
*
*
NaCl
0.5 mg/ml
1 mg/ml
E
NaCl
Bleomycin 0.5 mg/ml
Bleomycin 1 mg/ml
F
Relative number of myofibroblasts
3
2
1
0
*
*
NaCl
0.5 mg/ml
1 mg/ml